A New Approach to Antithrombotic Therapy — Evaluation of Combined Therapy of Thromboxane Synthetase Inhibitor and Very Low Dose of Aspirin

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SUMMARY The effect of a selective thromboxane (TX) synthetase inhibitor (OKY-046), alone and in combination with a very low dose of aspirin, on the platelet function was studied in healthy and diseased subjects.

A single dose of 100 mg OKY-046 was orally administered to patients with ischemic cerebrovascular disease (CVD) and healthy volunteers. TXB2 generation and platelet aggregation were measured before and at 1, 4, 6 and 8 hr after dosing. In addition, after the administration of a very low dose of aspirin (0.1-0.25 mg/kg/day) for at least one month, a single dose of OKY-046 was given to CVD patients. TXB2 generation and platelet aggregation were measured in the same manner as OKY-046 alone.

The effect of OKY-046 on platelet aggregation induced by arachidonic acid (AA) was different in each subject whereas platelet TXB2 generation was almost completely inhibited in all of the patients and healthy volunteers. OKY-046 had a slight inhibitory effect on collagen induced aggregation. A combination of OKY-046 with a very low dose of aspirin, on the other hand, produced additional inhibition of the platelet aggregation induced by both AA and collagen.

We, therefore, believe that the combined use of TX synthetase inhibitor and a very low dose of aspirin may provide a new approach to antithrombotic therapy without the inhibition of prostacyclin production.

Methods

Subjects The subjects consisted of 2 healthy volunteers (2 male; aged 30-32 yr; 62-67 kg) and 6 patients (5 male; 1 female; aged 54-63 yr; 48-72 kg) with ischemic cerebrovascular disease (CVD) in a chronic stage (table 1). None had received drugs known to interfere with platelet function during the preceding two weeks. Informed consent was obtained from each individual prior to the study.

Study Design In the first phase of this study, the effect of a single dose of 100 mg OKY-046 on platelet aggregation and TXB2 generation was investigated in all subjects before and 1, 4, 6 and 8 hr after dosing.

In the second phase, the combined effect of 100 mg OKY-046 and a very low dose of aspirin was investigated in the 6 CVD patients. Prior to the combined use of the OKY-046 and a very low dose of aspirin, the effect of a very low dose of aspirin on platelet cyclooxygenase activity was studied as a control. These patients received 0.1-0.25 mg/kg/day of aspirin orally for at least one month before OKY-046 was given. TXB2 generation and platelet aggregation were measured before and after the administration of OKY-046.

The present results suggest that 1) the accumulation and metabolism of cyclooxygenase products that accumulate when TX synthetase is blocked, differ in each subject, 2) additional inhibition is caused by the combined use of TX synthetase inhibitor and a very low dose of aspirin because the very low dose of aspirin partially reduces the proaggregatory cyclooxygenase products that accumulate when TX synthetase is blocked.

We, therefore, believe that the combined use of TX synthetase inhibitor and a very low dose of aspirin may provide a new approach to antithrombotic therapy without the inhibition of prostacyclin production.
xygenase activity was monitored by the measurement of TXB₂ generation after 0.1 mg/kg/day aspirin ingestion for one month. If platelet cyclooxygenase activity was not suppressed less than 70% compared to control, aspirin dosage was increased until that was suppressed less than 70%. Following the check of platelet cyclooxygenase activity, a single dose of 100 mg OKY-046 was given concomitantly. Platelet aggregation and TXB₂ generation were measured in the same manner as OKY-046 alone.

**Blood Sampling and Preparation of Platelet Suspensions**

Peripheral venous blood was collected in polyethylene syringes containing one tenth volume of 3.8% sodium citrate. The blood was carefully mixed, transferred to plastic tubes and centrifuged for 10 min at 200 × g at room temperature to obtain platelet rich plasma (PRP). Platelet poor plasma (PPP) was prepared by centrifugation of the remaining blood for 30 min at 2000 × g at room temperature. The platelet count in the PRP was adjusted to 2.5 × 10⁵/μl with autologous PPP. Homogenised platelets were obtained by repeated (3 times) freezing in dry ice-acetone and thawing.

**Platelet Aggregation**

Platelet aggregation was studied in 250 μl of PRP according to the method of Born⁰ by using a 4 channel aggregometer (Rikadenki, Japan). Arachidonic acid (AA) (2.0 mM: Sigma Co., USA) and collagen (3.0 μg/ml: Hormchemie Munich FRG) were used as the aggregating agents. Following one min preincubation, the aggregating agent was added to PRP and the change in light transmission was recorded continuously for 5 min. The maximal rate of aggregation was estimated as % light transmission from the steepest part of the curve.

**TXB₂ Generation**

TXB₂ generation was measured in connection with the platelet aggregation study. After 5 min of aggregation study, an aliquot of PRP (200 μl) was removed from the cuvette, mixed with 800 μl of cold phosphate buffer, containing 10⁻⁴ M indomethacin, and frozen in dry ice-acetone. TXB₂ generation from the homogenised PRP was measured using 1.0 mM AA in the same sequence as that of TXB₂ generation in intact platelets.

<table>
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<tr>
<th>Case No.</th>
<th>Sex</th>
<th>Age</th>
<th>Body weight (kg)</th>
<th>Diagnosis</th>
<th>CT findings</th>
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<td>72</td>
<td>CI</td>
<td>Small LDA (+)</td>
<td>Wall irregularity</td>
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</tbody>
</table>

Abbreviations: CI, cerebral infarction; LDA, low-density area; ICA, internal carotid artery.

Platelet cyclooxygenase and TX synthetase activities were determined by TXB₂ generation of the homogenised PRP. TXB₂ generation was measured by a radioimmunoassay technique¹³ and the results were expressed as ng/5 min/2.5 × 10⁷ platelets.

**Statistics**

All results are reported as the mean ± S.D. Statistical analysis was assessed by the Wilcoxon matched-pairs test.

**Results**

**Effect of OKY-046 Alone**

As shown in figure 1, the effect of OKY-046 alone on platelet aggregation induced by AA was different in each subject, though there was a statistically significant change in mean platelet aggregation following the administration of OKY-046. We classified the subjects into three groups according to the response of AA (2.0 mM) aggregation to OKY-046: type 1 = marked inhibition (% light transmission ≤ 15%), type 2 = reversible aggregation with moderate inhibition (% light transmission >15%), type 3 = irreversible aggregation. The number of type 1, type 2 and type 3 were 4, 2 and 2, respectively. Collagen (3.0 μg/ml) induced platelet aggregation was suppressed slightly from the predosing value of 82 ± 3% to 69 ± 8% by administration of OKY-046, as shown in figure 1. The effect of OKY-046 alone on TXB₂ generation from homogenised platelets and intact platelets are shown in figure 2. TXB₂ generation from intact platelets or homogenised platelets fell significantly by administration of OKY-046 at any points. The peak inhibition of TXB₂ generation did not differ among any of the subjects.

**Effect of OKY-046 Combined with very Low Dose of Aspirin**

A preliminary study was performed to determine the dose of aspirin. Although 0.1 mg/kg/day aspirin for one month showed over 50% inhibition on the platelet cyclooxygenase activity in 2 cases (cases No. 3, 5), a larger dose of aspirin was needed in the other 2 cases (cases No. 7, 8). For example, 0.25 mg/kg/day aspirin for one month was needed in case 7. The final dose of aspirin and effect of a very low dose of aspirin on TXB₂...
generation from homogenised platelets are shown in table 2. Although AA-induced platelet aggregation was suppressed significantly from the predosing value of 75 ± 6 to 46 ± 36% by a very low dose of aspirin, collagen induced platelet aggregation showed no significant change. The effect of OKY-046 combined with a very-low-dose of aspirin on platelet aggregation is shown in figure 3. AA-induced platelet aggregation was suppressed significantly from 46 ± 36% during very-low-dose aspirin treatment to 14 ± 20% after combined use of OKY-046 and very-low-dose aspirin. In addition, platelet aggregation of case 5, who showed no inhibition on AA-induced platelet aggregation by OKY-046 alone, was suppressed by the combined use of OKY-046 and very-low-dose aspirin. In collagen induced aggregation, combined therapy of OKY-046 and very-low-dose aspirin not only produced significant inhibition from 74 ± 7% during very-low-dose aspirin treatment to 51 ± 20% after the combined therapy, but also produced a significant decrease from 72 ± 5% after OKY-046. TXB₂ generation in intact platelets or homogenised platelets was also suppressed completely after the combination therapy, as shown in figure 4.

**Discussion**

The purpose of the present study was to determine the effect of a selective TX synthetase inhibitor alone and in combination with a very low dose of aspirin on the platelet function in patients with CVD.

First, we quantitated the effect of OKY-046 alone. The effect of OKY-046 alone on platelet aggregation induced by AA differed in each subject, whereas platelet TXB₂ generation was almost completely inhibited in all of the subjects. We classified the subjects into three groups in accordance with the response of AA-induced aggregation to OKY-046. TX synthetase inhibitors are known to fail to inhibit AA-induced platelet aggregation in PRP from some donors, designated "non-responder." It is considered that type 1 is "responder" and type 3 is "non-responder", respectively. In our study, a group showing a moderate inhibition of AA-induced aggregation was found. Some reports suggest that PG endoperoxides or other cyclooxygenase products may be sufficient to induce aggregation by AA, when TXA₂ synthesis is inhibited. Therefore, our results suggest that the accumulation and metabolism of pro-aggregatory metabolites differed in each subject. It is unknown, however, whether the diseased condition affects the response to OKY-046. In our findings, we could not find the relationship between the presence of CVD and the response of AA induced aggregation to OKY-046. Minno et al. have reported that it is impossible to determine the exact
Therefore, it is suggested that an additional effect on inhibit platelet aggregation and this combination ther-
beneficial effect in preventing of thrombus formation. Collagen may play a
absence of TXA2 generation. Collagen may play a
vivo thrombus formation because AA would not initi-
ated in vivo thrombus formation physiologically.
fore, that low-dose aspirin reduces PG endoperoxides
with a very low dose of aspirin may be of value as a new approach to antithrombotic therapy.

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